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Staphylococcal enterotoxin-B (SEB) is a common enterotoxin that can cause diarrhea and death in man. In these studies we have developed a specific and sensitive assay for the detection of SEB (enzyme linked receptor-based immunodot) in human fluids, plasma and urine. Our structure/function studies have revealed that amino acid sequence (130-160) of SEB (peptide #9) imparts toxic effects including cell death in PT cells. In addition, we found that SEB can activate neutral sphingomyelinase (N-SMase) resulting in the hydrolysis of sphingomyelin to ceramide and phosphocholine. Ceramide, in turn induces programmed cell death (apoptosis). Interestingly, several of the SEB mutants of peptide #9 were found to abrogate SEB toxicity in human kidney cells. Our findings will of potential value for the food industry, and to help determine toxemia in our soldiers. Such studies will also elaborate the pathophysiology of SEB induced toxemia in man.

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Subrata Chatterjee

PI - Signature Date

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Introduction: We have previously shown that glycosphingolipids may serve as putative receptor for Staphylococcal enterotoxin-B (SEB) (1-3). Accordingly, during the course of the study this year, we have attempted to develop a suitable immunodetection assay for SEB in human fluids, i.e., human plasma and urine. Further studies were performed to determine the biochemical mechanism of action of SEB in cultured human proximal tubular cells, namely apoptosis.

Methods and Results: A rapid, simple, and inexpensive sandwich enzyme-linked receptor based immunodot assay was developed for the detection of Staphylococcal enterotoxin-B (SEB) in human fluids by using purified glycosphingolipid digalactosylceramide (diGalCer) receptor for SEB. Three microgram of diGalCer was immobilized on a polyvinyledene secondary alkaline-phosphatase, the membrane was subsequently incubated with primary and secondary alkaline phosphatase labeled antibodies. A positive reaction was discerned as a blue spot. As little as 1 ng/ml of SEB could be detected in this assay. SEB did not bind to structurally related glycosphingolipids, such as glucosylceramide, galactosylceramide, and lactosylceramide in this assay. Of five monoclonal anti-SEB antibodies and commercial anti-SEB antiserum tested, latter was the most sensitive in our assay. The specificity of SEB assay was assessed by comparison with structurally related toxins, e.g., Staphylococcal enterotoxin-A, and toxic shock syndrome toxin 1 (TSST-1). TSST-1 was not detected in the assay. Only at very high concentration of SEA some cross-reaction was found (4).

In conclusion, we believe that this assay may be widely applicable because it is highly specific for SEB, it does not require special equipment, and the results can be obtained within a few hours with the naked eye. Since the receptor for SEB has a long-shelf life under adverse conditions, it can be easily stored and used for a long time (Appendix #1).

Structure/function relationship of SEB binding to human kidney cells. In our previous studies, we found that SEB peptide sequence KKKVTAQEL may be important in SEB induced cell proliferation and may be crucial in developing a neutralizing antibody to SEB. The binding of peptide sequence 93-112 in PT cells was on the order of 7-fold less than peptide sequence 130-160 (peptide #9), and 24-fold less than peptide sequence 191-120 (peptide #12). These studies have been reported previously. Since peptide #9 exerted a concentration-dependent inhibition of cell proliferation, and since scientists at Walter Reed (5,6) have prepared numerous mutant peptides of this particular peptide (Table I below). We found that when mouse T-and B lymphocytes containing monocytes at the ratio of 3:1 were incubated with SEB with a non-toxic dose on the order of 100% (Appendix 1; Fig. 1A), 50% (Fig. 1B) and 20% (Fig. 1C), respectively, several of these SEB mutants were able to abrogate SEB toxicity completely in the mouse cells, and partially in the human cells. Our preliminary studies indicate a 6-fold decrease in PT cell proliferation with wild type peptide #9 compared to control. In contrast, some amino acid substituents of peptide #9 surrounding the sequence, KKKVTAQEL (Table I below), did not impart such an effect. Further studies on these peptides in PT cells and *in vivo* in kidney are essential to provide a rational basis for SEB therapy in man.

Toxin Designation	AA Sequence	[^{3}H]-Thymidine Incorporation (cpm/well, N=6)
Wild type peptide #9	S-I-T-V-R-V-F-E-D-G-K-N-L-L-S-F-D-V-Q-T-N-K-K-K-V-T-A-Q-E-L	539 ± 50
Amino acid substituents	K-N-L-L-S-F-D-V-Q-T-N-K-K-K-V-T-A-Q-E-L-D	544 ± 48
of peptide #9	F-D-V-Q-T-N-K-K-K-T-A-Q-E-L-D	610 ± 52
	S-F-D-V-Q-T-N-K-K-K-V-T-A-Q-E-L-D-Y-L-T-R-H-Y-L-V-K-N-K-K-L-Y	588 ± 56
	T-N-K-K-K-V-T-A-Q-E-L-D-Y-L-T-R	502 ± 90
	K-V-T-A-Q-E-L-D-Y-L-T-R-H-Y-L-V-K	485 ± 40
	G-K-N-L-L-S-F-D-V-Q-T-N-K-K-K-V-T	4,386 ± 350
	P-H-A-G-L-K-K-K-K-S-V-T-V-L-D-V-G-D-A-Y	1,795 ± 110
	N-D-I-N-S-H-Q-T-D-K-R-K-T-C-M-Y-G-G	685 ± 46
	S-I-T-V-R–V-F-E-D-G-K-N-L-L-S-F-D-V-Q-T-N-K-K-K-V-T-A-Q-E-L	5,446 ± 390
	P-H-P-A-G-L-K-K-K-K-S-V-T-V-L-D-V-G-D-A-Y	4,974 ± 640
Control	No Toxin	3,151 ± 350

Table I: Amino acid substitution mutants of SEB peptide #9.

Neutral Sphingomyelinase (N-SMase) and Apoptosis

N-SMase has been implicated in a variety of cell systems to mediate the effects of cytokines such as TNF-a, IL-1, and interferon-γ. The basic mechanism may involve the binding of these cytokines to receptors. This in turn activates the N-SMase. N-SMase then cleaves sphingomyelin to ceramide (7-8). Ceramide, in turn, stimulates programmed cell death (apoptosis) presumably by activating nuclear factors, such as NFkB, p^{53}, bcl_2-bax and the ICE family (9). Our laboratory has purified the human N-SMase (10). We have also recently completed the molecular cloning of N-SMase and expression in mammalian cell lines. Expression of N-SMase in human leukemic (HL-60) cells resulted in spontaneous apoptosis and this phenomenon was abrogated by antibody against N-SMase (11). Moreover, TNF-α induced apoptosis was also abrogated by antibody against N-SMase as determined by FITC-conjugated Annex-V propidium iodide staining and FACS-analysis (Fig. 1).

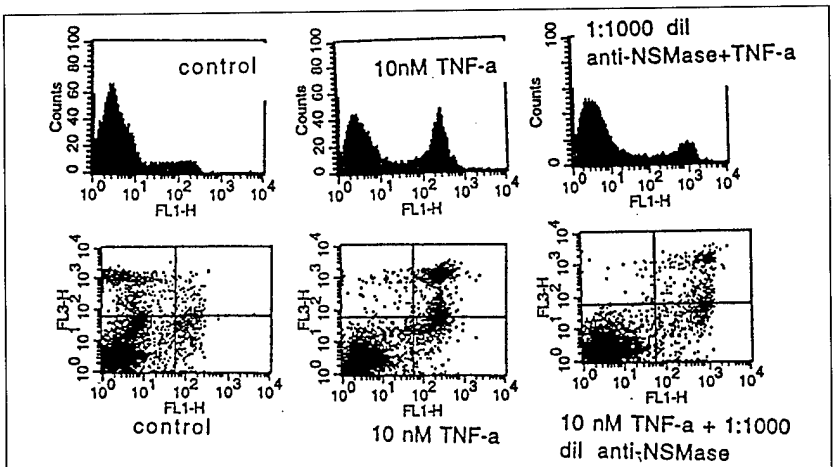

Fig.1: Effects of TNF-α and N-SMase antibody on apoptosis in HL-60 cells.

We have also observed that the biological responses of PT cells concomitant to binding of SEB are: release of lactate dehydrogenase, cell death and the production of nitric oxide. The latter finding concurs with pathophysiological observations of SEB-induced shock and dilation of blood vessels in monkey kidney (12). Furthermore, our studies indicate that the SEB/SEB peptide #9 mediated toxic effects in cultured PT cells may involve activation of N-SMase, the production of ceramide and apoptosis (DNA ladder formation) (Fig. 2A,B,C). Whether amino acid substituents of SEB peptide #9 may abrogate the toxic effects of SEB and/or SEB peptide #9 are not known. Such studies will elaborate the patho-physiology of SEB induced toxemia in man, and will establish whether or not altered-peptide #9 or gene constructs serve as potential therapeutic agents and/or vaccine candidates in experimental animals and in man.

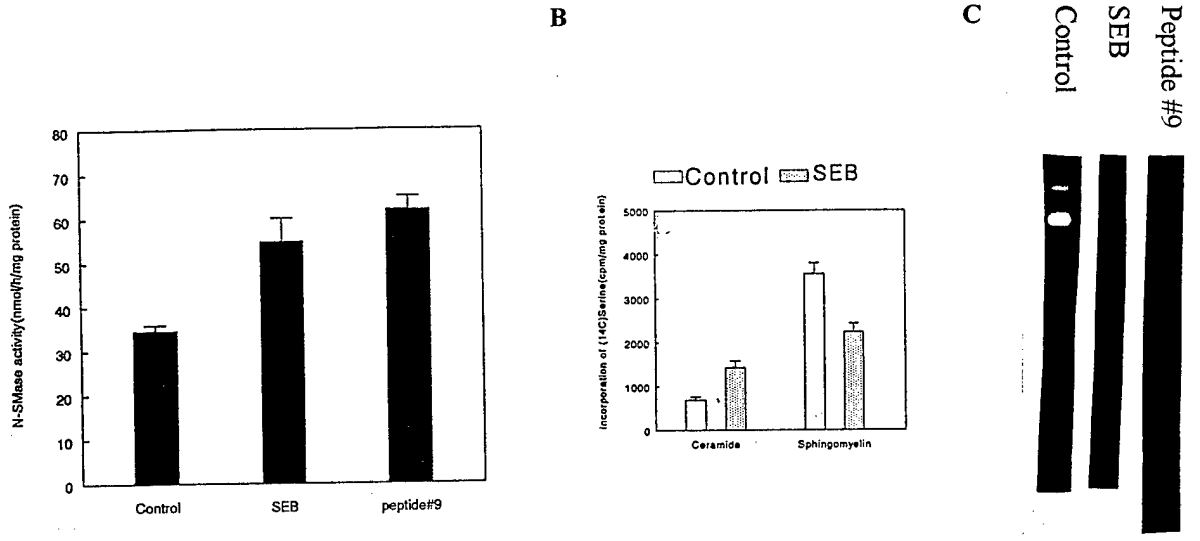

Fig. 2A,B,C Effects of SEB and peptide #9 (10 μg/ml, 15 min, 37°C) on N-SMase activity (A), ceramide/ sphingomyelin level (B) and apoptosis (DNA laddering; C).

In this experiment, confluent cultures of PT cells were incubated with serum-free Eagles minimum essential medium containing 1 μg/ml cycloheximide, SEB (10 μg/ml) and SEB peptide #9 ((S-I-T-V-R-V-F-E-D-G-K-N-C-C-S-F-D-V-Q-T-N-K-K-K-V-T-A-Q-E-L). After incubation for 24 hr, cells were harvested, stained with propidium iodide and counter-stained with FITC conjugated Annexin-V. The stained samples were next subjected to FACS analysis.

Our studies revealed that in control PT cells only 0.07% cells were necrotic and 0.02% cells were apoptotic (Fig. 1;4). In contrast, SEB markedly induced apoptosis in these cells. For example, 61.6% of cells incubated with SEB were apoptotic, 6% were necrotic and the rest were normal (Fig. 2,4). Similarly, cells incubated with peptide #9 were 54% apoptotic, 7.1% necrotic and the rest were normal (Fig. 3,4).

In summary, our additional data suggests that SEB/SEB peptide #9 mediated toxic effects in human PT cells may involve apoptosis via the sphingomyelinase pathway.

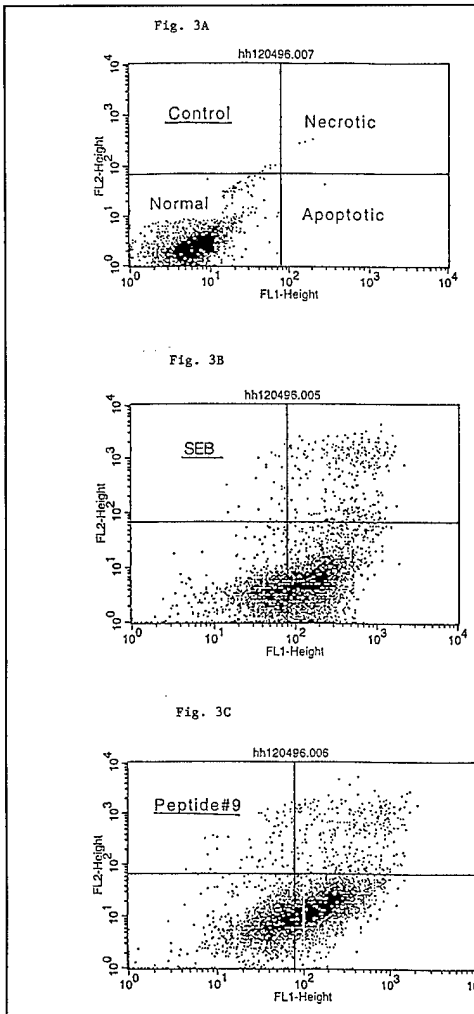

Fig. 3 FACS analysis of apoptotic human kidney proximal tubular cells after staining with Annexin-V and propidium iodide. Cells were incubated in the presence of cyclohemiximde (1 μg/ml); control, Fig. 3A), Staphylococcal enterotoxin-B (10 μg/ml) (Fig. 3B), and SEB peptide #9 (Fig. 3C) for 24 hr, and subjected to dual parameter analysis following staining with Annexin-V, propidium iodide, and computerized analysis of percent distribution of apoptotic, necrotic and normal cells.

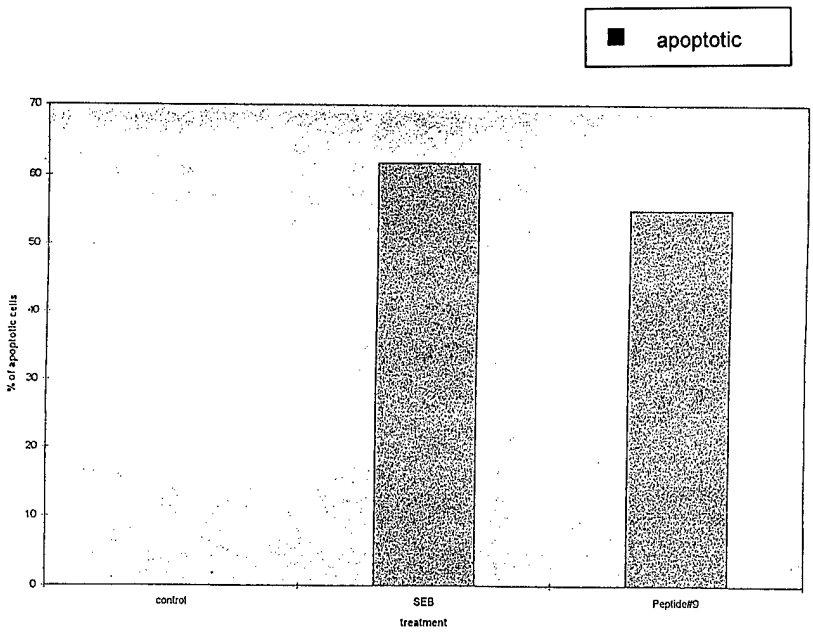

Fig. 4 Effect of SEB on kidney cell proliferation. The data shown above was subjected to computer assisted calculation of percent apoptosis of cells incubated with or without SEB and peptide #9.

Conclusions: During this period, we have developed a suitable and sensitive assay for detecting SEB in human fluids. Our studies on mutant peptides representing the 130-160 amino acid sequence of SEB provided additional novel information regarding structure/function relationships of SEB induced toxemia in man.

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Appendix #1

ANALYTICAL BIOCHEMISTRY 245, 213–217 (1997)
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A Receptor-Based Immunoassay to Detect *Staphylococcus* Enterotoxin B in Biological Fluids

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A rapid, simple, and inexpensive sandwich enzyme-linked receptor based immunodot assay was developed for the detection of staphylococcal enterotoxin B (SEB) in human fluids by using purified glycosphingolipid digalactosylceramide (diGalCer) receptor for SEB. Three micrograms of diGalCer was immobilized on a polyvinyledene difluoride membrane and the membrane was subsequently incubated with primary and secondary alkaline-phosphatase-labeled antibodies. A positive reaction was discerned as a blue spot. As little as 1 ng/ml of SEB could be detected in the assay. SEB did not bind to structurally related glycosphingolipids, such as glucosylceramide, galactosylceramide, and lactosylceramide in this assay. Of five monoclonal anti-SEB antibodies and commercial anti-SEB antiserum tested, the latter was the most sensitive in our assay. The specificity of SEB assay was assessed by comparison with structurally related toxins, for example, staphylococcal enterotoxin A, and toxic shock syndrome toxin 1 (TSST-1). TSST-1 was not detected in the assay. This was because these toxins were not recognized by the anti-SEB antibody and did not bind to diGalCer. In conclusion, we believe that this assay may be widely applicable because it is highly specific for SEB, it does not require special equipment, and the results can be obtained within few hours with the naked eye. Since the receptor for SEB has a long shelf life, it can be easily stored and used for a long time.

Infections caused by staphylococci remain an important cause of morbidity and mortality. The virulence factors associated with the toxinogenic diseases of *Staphylococcus aureus* are the staphylococcal enterotoxins. Among them staphylococcal enterotoxin B (SEB)[2] attracts the most attention because of its implication in immunological reactions. SEB has been shown to be able to stimulate mitogenic activity in T-cells (1). This phenomenon appears to involve specific binding of the toxin to major histocompatibility complex (MHC) class II molecules and subsequent stimulation of the T-cell via the TCR-V-β elements (2).

Glycosphingolipids (GSL) are composed of carbohydrates, fatty acid, and sphingosine. They are components of the eukaryotic cell membrane. Recently, GSL have been implicated in various biological phenomena. For example, GSL have been shown to be involved in cell proliferation (3, 4), cell migration (5, 6), and apoptosis (programmed cell death) (7). Most importantly, GSL have been shown to serve as receptors for numerous bacterial toxins and viruses (8–10). For example, a ganglioside GM_1 has been long established to serve as a receptor for cholera toxin (11).

We have recently shown that digalactosylceramide (diGalCer) present in the human kidney and proximal tubular cells can specifically bind SEB. It did not bind structurally related toxins, staphylococcal enterotoxin A (SEA) and toxic shock syndrome toxin-1 (TSST-1) (12). The specificity of binding to the diGalCer receptor and physiological function was established subsequently. Therein, we found that SEB induced the uptake of [^{14}C]choline and increased the synthesis of phosphatidylcholine; in contrast, SEA and TSST-1 failed to stimulate phospholipid biosynthesis (13).

Since the above delineated an important role of the receptor in mediating the metabolic action of SEB, we rationalized and designed a rapid assay for this toxin employing receptor-based technology. We believe that this assay would be widely applicable because of the long shelf life of the receptor.

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[2] Abbreviations used: SEB, staphylococcal enterotoxin B; MHC, major histocompatibility complex; GSL, glycosphingolipids; diGalCer, digalactosylceramide; SEA, staphylococcal enterotoxin A; TSST-1, toxic shock syndrome toxin-1; PVDF, polyvinyledene difluoride; MAB, monoclonal anti-SEB antibodies; AS, antiserum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GlcCer, glucosylceramide; GalCer, galactoceramide; LacCer, lactosylceramide.

0003-2697/97 $25.00

TABLE 1

Structure of Glycosphingolipids Used in This Study

Glycosphingolipid	Structure
Glucosylceramide	Glcβ1–1Cer
Galactosylceramide	Galβ1–1Cer
Lactosylceramide	Galβ1–4Glcβ1–1Cer
Digalactosylceramide	Galα1–4Galβ1–1Cer

MATERIALS AND METHODS

Materials

Polyvinyledene difluoride (PVDF) membrane was obtained from Millipore Corp. (Bedford, MA). SEB, SEA, TSST-1, and monoclonal mouse anti-SEB antibodies (MAB), clones 2B, 3B, 6B, 12B, and 18B were obtained as a gift from Drs. Peter Gemski and Marti Jett (Washington, DC). SEB and SEA preparations were free of contaminants with other toxins, for example, lipopolysaccharides, lipid A. Upon gel electrophoresis SEB resolved as a single band having a molecular weight on the order of about 30 kDa. Anti-mouse and anti-rabbit alkaline-labeled polyclonal antibodies were from Boehringer Mannheim Corp. (Indianapolis, IN). Bovine serum albumin (initial fractionation by heat shock) was from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-SEB antiserum (AS) and other chemicals were also obtained from Sigma Chemical Co. Digalactosylceramide was prepared from human Fabry kidney and characterized as described previously (12).

Immunodot Blot Assay

The following procedure for the detection of SEB on PVDF membrane has been developed. Three micrograms of diGalCer dissolved in chloroform:methanol (2:1) was applied to 4-mm-diameter spots on PVDF membrane and dried at room temperature. Pieces of the membrane were placed to the wells of 96-microtiter plate and were incubated for 30 min in a 3% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature. Then it was washed three times with PBS and incubated in 400 μl of PBS spiked with SEB for 60 min at room temperature (all following incubations were performed in the volume of 400 μl). The membrane was washed three times with PBS and once with 3% BSA in PBS for 10 min. After the membrane was washed it was incubated with primary antibodies (anti-SEB diluted 1:40,000 or MAB diluted 1:1,000 with 3% BSA in PBS) for 2 h at room temperature. Then the membrane was washed three times with PBS and once with 3% BSA, and was incubated with alkaline phosphatase-labeled antibodies (diluted 1:1000 with 3% BSA in PBS) for 2 h at room temperature. After washing it was incubated in the solution of alkaline phosphatase substrate (100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$ (pH 9.5):5-bromo-4-chloro-3-indolyl phosphate:4-nitro blue tetrazolium chloride, 300:2:1) for 10 min in the dark. Reaction was stopped by washing with water.

Quantitation of Results

The quantitative comparison of staining was performed by scanning densitometry on a Protein DNA Imager (PDI Inc., Huntington Station, NY) equipped with image processing software.

RESULTS AND DISCUSSION

This study elaborated a novel approach to detect small quantities of SEB in mammalian fluids employing a receptor based immunodot assay.

We have determined the specificity of binding of SEB to diGalCer and structurally related GSL, such as glucosylceramide (GlcCer), galactosylceramide (GalCer), and lactosylceramide (LacCer). The chemical structure of these GSLs is summarized in Table 1. We have used a MAB 12B at a dilution 1:1,000. As shown in Fig. 1, SEB at a concentration of 1 μg/ml did not significantly bind to GlcCer, GalCer, and LacCer but produced strong binding to diGalCer. These results confirm our previous report on the binding of [^{125}I]SEB to various glycosphingolipids immobilized on a microtiter plate

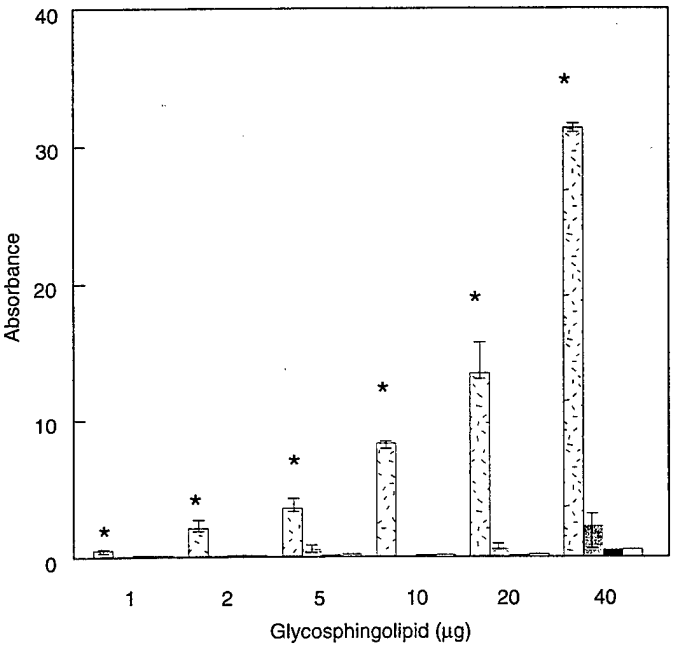

FIG. 1. Specificity of binding of SEB (1 μg/ml in PBS) versus control (no SEB) to various glycosphingolipids. MAB 12B at a dilution of 1:1,000 was employed. ⊠, diGalCer; □, GlcCer; ■, GalCer; □, LacCer. Values are means of three experiments ±SD. *Significant difference from control, $P < 0.05$ (Student's t test).

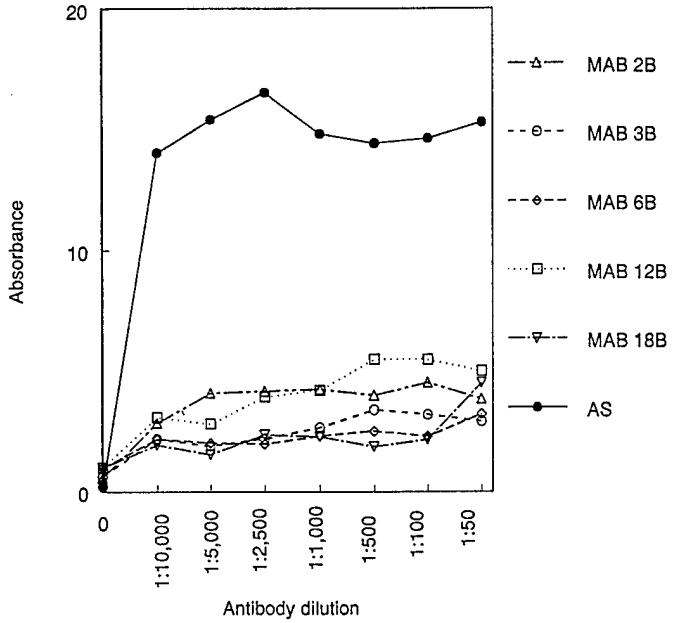

FIG. 2. Effect of antibody dilution on the immunodetection of SEB (1 μg/ml in PBS). The amount of diGalCer was 3 μg.

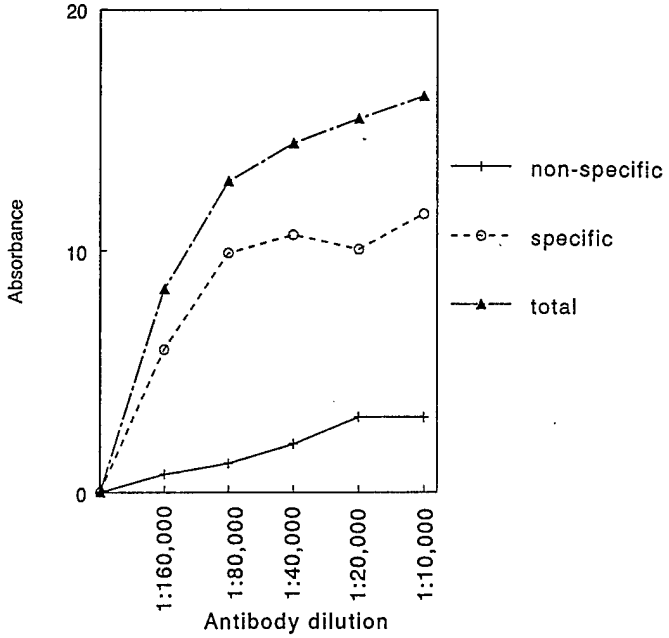

FIG. 3. Binding of different dilutions of anti-SEB AS to the complex diGalCer–SEB (total) versus diGalCer alone (nonspecific). Specific curve was obtained by subtraction of nonspecific values from total values.

(12). An additional advantage of our current method is the use of nonradioactive reagents to detect SEB.

The effect of an antibody dilution on the immunodetection of SEB is shown in Fig. 2. Polyclonal anti-SEB AS even at a dilution 1:10,000 was much more sensitive (sevenfold) than the monoclonal antibodies in detecting SEB.

Polyclonal anti-SEB AS obtained from a commercial source bound to the complex diGalCer–SEB (concentration of SEB was 1 μg/ml) in a saturable fashion. However, nonspecific binding of anti-SEB AS to diGalCer (10 μg) in this assay increased with increase in the concentration of the AS (Fig. 3). Accordingly, in subsequent studies we have chosen the AS dilution of 1:40,000 to decrease the nonspecific binding and at the same time to retain sensitivity. Similarly, we have determined that the minimal concentration of diGalCer that retains the sensitivity but eliminates nonspecific binding of AS (at the dilution 1:40,000) is 3 μg per assay.

Next, we studied the detection of different concentrations of SEB with MAB (diluted 1:1,000) and AS (diluted 1:40,000). We found that the commercial anti-SEB AS is most sensitive in the assay at all concentration ranges of SEB (Fig. 4). However, at higher concentrations of SEB (2 μg/ml), it is feasible to use MAB when a definitive result on the nature of intoxication is desired.

We have also assessed the possible interference of structurally related toxins, SEA and TSST-1, with our assay employing diGalCer and commercial anti-SEB AS. At low concentrations (0.5–1 μg/ml), SEA and TSST-1 could not be detected in our assay (Fig. 5). Only at very high concentrations of SEA (>1 μg/ml) could it be nonspecifically detected in the assay. These findings suggest that our assay may be suitable for use in de-

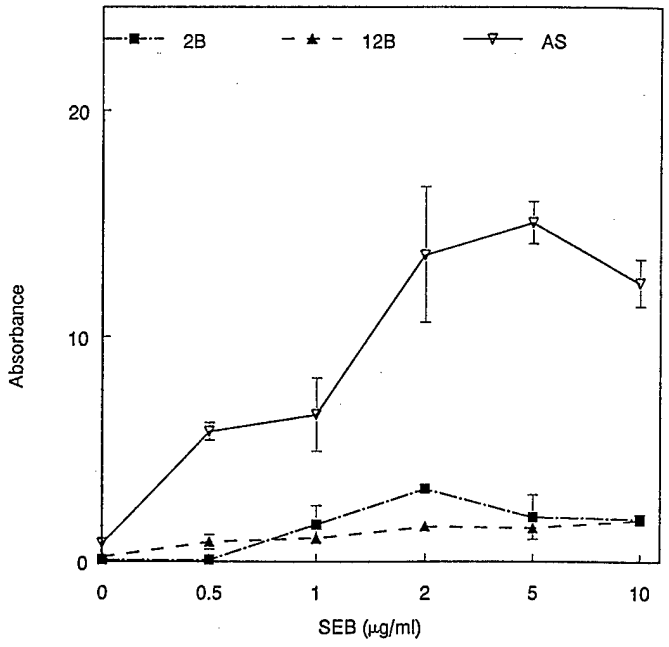

FIG. 4. Immunodetection of different amounts of SEB with monoclonal antibodies 2B, 12B (1:1,000), and antiserum (1:40,000). Values are means of three experiments ±SD.

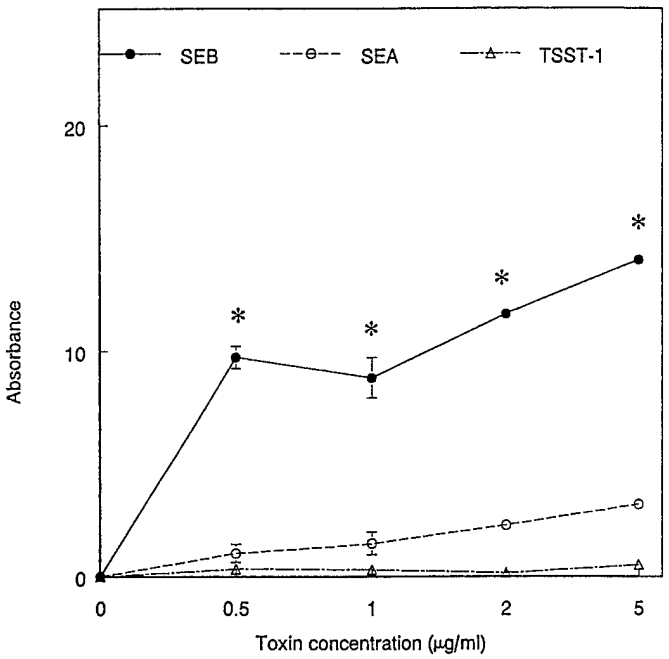

FIG. 5. Immunodetection of structurally related toxins in spiked PBS with anti-SEB AS after binding to diGalCer (3 μg). Values are means of three experiments ±SD. *Significant difference from SEA, $P < 0.05$ (Student's t test).

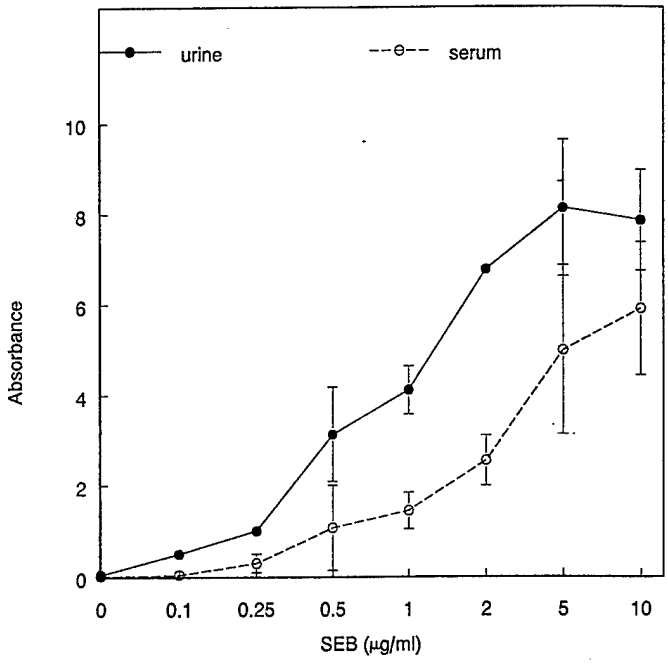

FIG. 6. Immunodetection of SEB either in spiked urine or in spiked serum with anti-SEB AS. Values are means of three experiments ±SD.

tecting SEB in samples contaminated with other structurally related toxins. However, for samples containing high concentrations of SEA (>1 μg/ml) and a low concentration of SEB (<0.1 μg/ml) an alternative method may be recommended. Nevertheless, the present results are consistent with our previous reports demonstrating that SEA and TSST-1 did not compete for binding with SEB in our microtiter plate assays and in cultured human kidney proximal cells (12). The latter was accompanied by a marked stimulation of phosphatidylcholine synthesis by SEB but not by SEA and TSST-1 (13).

To study the possibility of measuring SEB with commercial anti-SEB AS in mammalian biological fluids, we used human urine and serum spiked with SEB (100–10,000 ng/ml). The detectability of SEB in biological fluids was lower than that in PBS. The concentration of SEB that could be detected in the serum in our assay was 500 ng/ml. At the same time 100 ng of SEB per milliliter in the spiked urine could be easily detected with anti-SEB AS (Fig. 6). The recovery of the enterotoxin from spiked urine was about 80% of the amount added compared with spiked PBS. Subsequently, we found that as little as 1 ng/ml of SEB in spiked urine was detectable employing our assay (Fig. 7). However, 20 ng of SEB per milliliter of urine was the lowest amount measured that was statistically significant. In the case of very low concentrations of toxin, an overnight incubation with primary anti-SEB antibodies may improve the sensitivity of the assay (data are not shown). Our experiments do not explain why the sensitivity of the assay for SEB in human urine is higher than in human serum. A possible reason may be that serum has a number of lipid-binding proteins

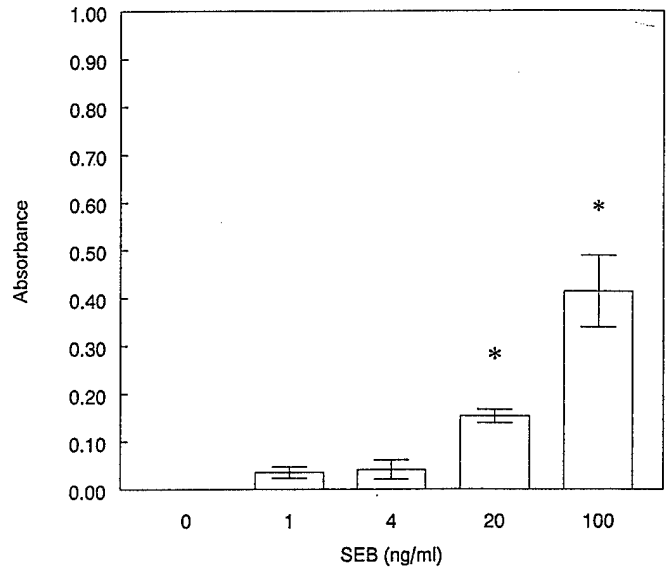

FIG. 7. Immunodetection of SEB in spiked urine with anti-SEB AS. Values are means of three experiments ±SD. *Significant difference from control (no SEB), $P < 0.05$ (Student's t test).

including albumin that may bind to diGalCer, thus preventing it from interacting with SEB.

In conclusion, various procedures have been developed to detect staphylococcal enterotoxins in food (14–21). Such methods employ microslide immunodiffusion assay, passive and reversed passive hemagglutination or latexagglutination assay, radioimmunoassay, enzyme-linked immunosorbent assay, and skin test assay. However, such studies have often suffered from nonspecific binding (22). Our assay is designed to specifically capture SEB to its receptor followed by immunodetection. Our method is a fast, simple, and inexpensive immunodot assay to detect specifically SEB in biological fluids. This analysis does not require special equipment and the results can be obtained within few hours with the naked eye. The minimal sensitivity of the assay for SEB (1 ng/ml) is comparable to that of existing tests (23).

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Staphylococcal Enterotoxin-B Binds to Digalactosylceramide and Alters [^{14}C]-Choline Transport in Cultured Human Kidney Proximal Tubular Cells

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Key Words
glycosphingolipids (GSL), proximal tubular cells (PT), Staphylococcal enterotoxin-B (SEB), phosphatidylcholine

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Abstract

We have characterized a glycosphingolipid receptor for Staphylococcus enterotoxin-B(SEB) in cultured human kidney proximal tubular (PT) cells. Solid-phase binding of ^{125}I-SEB to the glycosphingolipid receptor was concentration-dependent and was not displaceable by two structurally related toxins, such as Staphylococcal enterotoxin-A (SEA) and toxic shock syndrome toxin-1(TST-1). Rat kidney cells did not bind ^{125}I-SEB. However, when the rat kidney cells were pre-incubated with digalactosylceramide there was a concentration-dependent binding of ^{125}I-SEB.

We also studied the effects of SEB on [^{14}C]-choline transport and metabolism of choline containing phospholipids in these cells. SEB increased the uptake of [^{14}C]-choline in PT cells as a function of toxin concentration, incubation time, and pH. The maximum increase in uptake (3.5-to-5-fold compared to control) was observed at a toxin concentration of 10 ug/10^4 cells, at 4 hr, and at pH 7.4. In contrast, SEA and TST-1 failed to alter [^{14}C]-choline uptake in PT cells.

Maximum increase in the incorporation of [^{14}C]-choline into phosphatidlycholine (3-fold compared to control) was observed at 4 hr after incubation with SEB. The cellular level of phosphatidylcholine was also increased (2-fold compared to control) in PT cells incubated with SEB. This was accompanied by a 3-to-4-fold increase in CTP: phosphocholine,cytidyl-transferase activity.

In sum, SEB specifically binds to digalactosylceramide and stimulates the uptake of [^{14}C]-choline and phosphatidylcholine synthesis in PT cells via activating CTP: phosphocholine, cytidyltransferase, or both.

Introduction

Staphylococcus aureus secretes at least five somewhat structurally homologous toxins (termed A-E). Among them, Staphylococcal entertoxin-B (SEB)is one of the most intensively studied toxins and its crystal structure has only recently been described (1-7). Ingestion of food contaminated with such bacteria is the most common mode of transmission into the host. After uptake by the host cells, the pathophysiological sequel of SEB begin, including vomiting, diarrhea, and death. This toxin was shown to bind to the V.B. sequence in lymphocytes responsible for profound immunological response (1). However, previous studies also showed that more than 75% of the ^{125}I-SEB injected intravenously into monkeys and rabbits was associated with the kidney (8,9). Moreover, ligation of renal artery

severely impaired the turnover of ^{125}I-SEB in these experimental animals (10,11). Interestingly, humans and rabbits are highly susceptible to SEB, whereas rats are not.

Glycosphingolipids (GSL) which are composed of sugars, fatty acids, and sphingosine are components of the eukaryotic cell membrane, have been accorded many biological functions (12,13). Most importantly, they have been shown to serve as receptors for various bacteria and viruses (14-19).

In this paper we present data that reveal that a human kidney digalactosylceramide serves as the receptor for SEB, and that this receptor may be involved in mediating SEB induced alterations in [^{14}C]-choline tranport and phosphatidylcholine synthesis.

Materials and Methods

Materials - ^{125}NaI (specific activity 561MBq per ng) was purchased from Amersham Life Science, Illinois. [methyl-[^{14}C]-choline chloride (specific activity 2.04 Gbq/mmol), CDP [U-[^{14}C]-choline (specific activity 2.1 GBq/mmol),and [^{14}C]-sucrose (specific activity 2.074 GBq/mmol) were purchased from Amersham Life Science, Arlington Heights, Illinois. All other chemicals were purchased from Sigma Chemical Company.

Cell culture - Human kidney cells (PT cells) were prepared as described previously (20). Human cadaver kidneys (post mortem $\leq$ 12 h) were used for the isolation of PT cells, and to purify the putative GSL receptor for SEB. Digalactosylceramide was prepared from the kidney of a Fabry's patient. Normal rat kidney cells were purchased from American Tissue Culture Collection (Rockville, Maryland). Cells (x 10^4) were seeded in 24 well trays and grown for six days in medium containing 10% fetal calf serum (Hyclone, Utah).

Lipid extraction and fractionation of glycosphingolipids from human kidney - Total lipids were extracted from freeze-dried cultured PT cells or human kidney cortex or both by vigorous homogenization and extraction with hot (50^{o}C) chloroform:methanol 2:1 (v/v), 10 ml/mg protein as previously described (21). The lipid extracts were pooled and dried by flash evaporation. Water-soluble contaminants were removed from the lipid extracts by using the procedure of Folch, Lees and Sloane-Stanley (22).

Quantitation of GSL- GSLs were quantified by high performance liquid chromatography (HPLC), as described (23) after perbenzoylation (24). An aliquot of perbenzoylated GSL sample was suspended in hexane and subjected to HPLC on a Spherisorb Si-5 column with detection at 230 nm. The amount of

GSL was calculated by using a standard curve for the respective GSLs.

Solid-phase binding assay- Solid phase binding assay on microtiter plates was carried out according to the method of Karlsson and Stromberg (25). Briefly, microtiter plates were coated with GSL (50 μl, suspended in methanol) by allowing it to evaporate at room temperature for overnight. The wells were incubated with 2% BSA in PBS for 2 h at room temperature and then rinsed twice with fresh solution. ^{125}I-SEB in PBS containing 2% BSA (5 x 10^5 cpm/well) was added to the wells, and incubation continued at room temperature for 4 h. The wells were washed six times with PBS containing 2% BSA and then dried, cut, and counted in a gamma spectrometer.

Incorporation of digalactosylceramide in normal rat kidney cells and studies on the binding of ^{125}I-SEB- Normal rat kidney cells x^4 were grown to confluence and pre-incubated with the various GSL suspended in lipoprotein deficient serum (0 to 60 nmole) for 1 h at 37°C. Next, the binding of ^{125}I-SEB was pursued for 2 h at 37°C. The cells were washed with PBS - 0.2% BSA, and then with PBS as described (19). The monolayers were solubilized in 1N NaOH over night. The cell extracts were solubilized in a small aliquot of water and used for the measurement of protein and radioactivity as described previously (26). The specific activity of ^{125}I-SEB used in these studies was 100 cpm/pg.

[^{14}C]-choline uptake studies- PT cell cultures were grown to confluency in 24-well trays or P-100 dishes in minimum essential medium containing 10% fetal bovine serum. Cells were incubated with SEB in medium-199 (0.3 ml) at 37°C and methyl-[^{14}C]-choline chloride (1 X 10^5 dpm/well) for the indicated times. The reaction was stopped by removing the medium and washing the cells with chilled phosphate-buffered saline. The cells were solubilized with 1 ml of 1N NaOH and [^{14}C] radioactivity and protein contents were measured (26).

Measurement of phospholipid content- The phospholipids were separated by TLC and eluted from the silica gel. The phosphorous content of the individual phospholipids was measured according to the method of Bartlett (27).

CTP:Phosphocholine cytidyltransferase activity measurement- CTP: phosphocholine cytidyltransferase activity in PT cells incubated with and without SEB was measured as described (28).

Discussion

Previously, glycosphingolipids have been implicated as receptors for bacterial toxins; for example, cholera and tetanus toxin receptors are known to be glycosphingolipids. Previously, the presence of GlcCer, GalCer, LacCer as well as globotriosylceramide and globotetraosylceramide, but not digalactosylcermide was found in rat kidney (29). Accordingly, we did not observe the binding of ^{125}I-SEB to rat kidney GSLs. However, upon the incorporation of digalactosylceramide in rat kidney cells there was a saturable binding of ^{125}I-SEB. Digalactosylceramide present naturally in human kidney may serve as a receptor for SEB, and may explain the significant role of kidney in SEB-induced toxemia in man.

Our results presented here confirm that SEA and TST-1 receptors do not share a similar domain with SEB, although they have some degree of structural homology. This could possibly be geared to the differences in the physiological actions of these toxins, which have been suggested to be determined by the nature of the receptor (30).

One of the most exciting findings in our study was a SEB specific increase in [^{14}C]-choline uptake in PT cells. We believe that this high order of specificity may be due to the presence of a SEB specific glycosphingolipid receptor in PT cells(19,31).

We observed a 3-to-4-fold increase in cytidyltransferase enzyme activity in PT cells incubated with toxin for two hours. This may explain the increased incorporation of [^{14}C]-choline into cellular phosphatidylcholine and an increase in the cellular levels of phosphatidylcholine upon incubation with toxin. Further work is needed to assess whether the observed increase in enzyme activity is due to an increase in the mass of enzyme or to direct activation(e.g.,phosphorylation of membrane-bound enzyme) by SEB.

We have previously observed that LPC exerts toxic effects in cells. Whether LPC mediated the SEB-induced toxicity in human kidney PT cells is unclear from our studies. Further studies are required to relate the SEB-mediated changes in [^{14}C]-choline transport with the toxic effects of SEB in human PT cells. In particular, it may be worth studying whether PC hydrolysis via phospholipases (involving SEB) may be involved in signal transduction processes (32) that may lead to cell death.

Conclusion

Effects of various glycosphingolipids on the binding of ^{125}I-SEB to purified receptor- The specificity of SEB binding to GSL was assessed by solid phase binding assays employing GalCer,

GlcCer, $GbOSe_3Cer$, $GbOSe_4Cer$, GM_3, GM_1, GT_1b and sulfatide (SO_4-GalCer) (*Table I*). None of these glycosphingolipids (1-1000 ng/well) bound ^{125}I-SEB (data not shown).

Table I: Structure of some glycosphingolipids in human kidney PT cells.

Glycosphingolipid	Structure
Sulfatide	SO_3-Galß1-1Cer
Galactosylceramide	Galß1-1Cer
Glucosylceramide	Glcß1-1Cer
Lactosylceramide	Galß1-4Glcß1-1Cer
Galabiosylceramide	Galα-4Galß1-1Cer
Globotriosylceramide	Galα1-4Galß1-4Glcß1-1Cer
Globotetraosylceramide	GalNAcß1-3Galα1-4Galß1-4Glcß1-1Cer
GM_3	NeuAc 2-3Galß1-4Glcß1-1Cer

Effects of structurally related toxins on the binding of ^{125}I-SEB to the purified receptor- Unlabeled SEB (concentrations of 1 ng and higher) almost completely displaced ^{125}I-SEB from binding to SEB-R (*Fig.1*). In sharp contrast, at a concentration range of 0-4 ng/well, SEA and TST-1 were unable to displace ^{125}I-SEB from binding to SEB-R (*Fig. 1*).

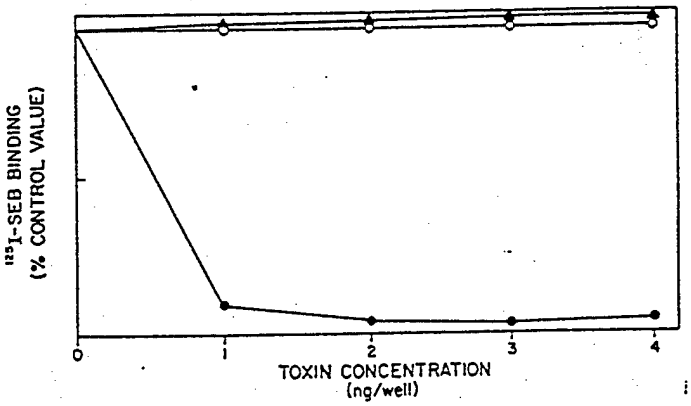

Fig. 1: Dose-dependent displacement (an inhibition) of ^{125}I-SEB binding to purified glycosphingolipid receptor (SEB-R). 20 ng of SEB-R/well was immobilized on plastic microtiter wells and the binding displacement of ^{125}I-SEB was measured in the presence of various concentrations of (●) SEB, (○) SEA and (▲) toxic shock syndrome toxin, as described under "**Materials and Methods**". Each point represents the mean of three experiments, measured in triplicate.

Effects of various glycosphingolipids on the binding of ^{125}I-SEB in cultured normal rat kidney cells- We found that normal rat kidney cells alone did not bind ^{125}I-SEB. Moreover, ^{125}I-SEB binding to these cells was not observed when preincubated with glucosylceramide, lactosylceramide, digalactosyldiglyceride, and globotrioslyceramide. Only following incubation of cells with human kidney digalactosylceramide there was a concentration-dependent, saturable binding of ^{125}I-SEB in these cells (Fig. 2).

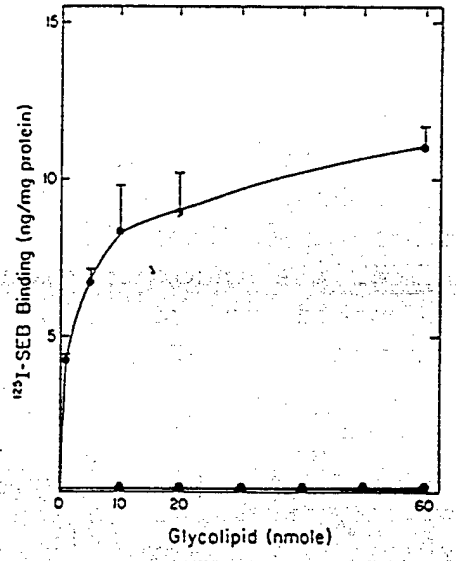

Fig. 2: Effects of various glycosphingolipids on the binding of ^{125}I-SEB in normal rat kidney cells. The bars represent actual data points: (●), digalactosylceramide; (○), digalactosyldiglyceride; (▲), lactosylceramide.

Effects of time of incubation with various toxins on the uptake of [^{14}C]-choline uptake in PT cells- Only SEB exerted a time-dependent increase in the uptake of [^{14}C]-choline in PT cells (Fig. 3). After incubation with SEB for 2 hr and 4 hr, the increase in the uptake of [^{14}C]-choline in PT cells was on the order of 300% and 500%, respectively, compared to control. In contrast, the two structurally related toxins, SEA and TST-1, did not increase the uptake of [^{14}C]-choline into PT cells, as compared to control.

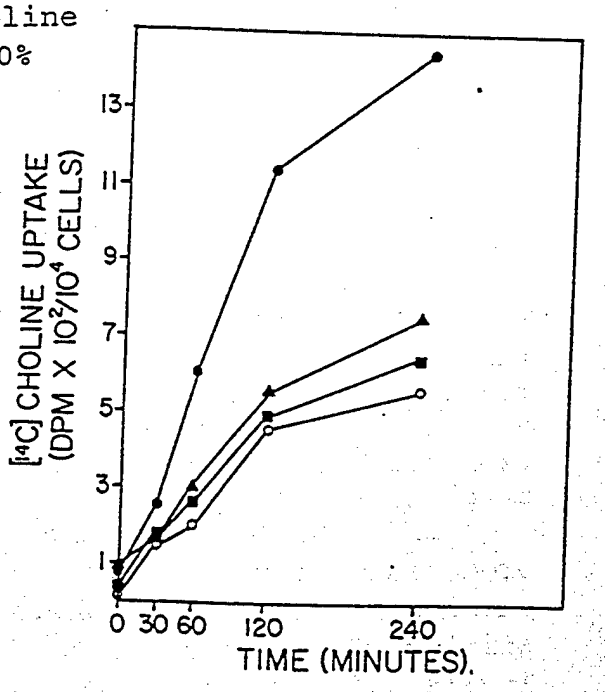

Fig. 3: Effects of time of incubation with various toxins on the incorporation of [^{14}C]-choline in PT cells. Cells were incubated with SEB (●); SEA (▲); TST-1 (■) and without toxin (○).

Effects of SEB on the incorporation of [^{14}C]-choline into cellular phospholipids- SEB did not alter the incorporation of [^{14}C]-choline in phosphatidylethanolamine derived from PT cells (Figure 4A). Although SEB did not alter the incorporation of [^{14}C]-choline into phosphatidylcholine after 30 min of incubation(Figure4B),it subsequently stimulated the incorporation of [^{14}C]-choline in phosphatidylcholine on the order of 200-250% compared to control. This effect was observed for up to 4 hr after incubation of cells with SEB. SEB gradually increased the incorporation of [^{14}C]-choline into sphingomyelin in PT cells. The maximum increases in the incorporation, 150% and 175% higher than control, were observed after 120 min and after 240 min, respectively, of incubation of cells with SEB, (Figure 4C). The effects of SEB on the incorporation of [^{14}C]-choline in lysophosphatidylcholine is presented in Figure 4D. We observed that at earlier time points (30 min and 60 min) SEB did not alter the incorporation of [^{14}C]-choline in LPC in PT cells, as compared to control. But, after 120 min and 240 min of incubation, SEB increased the incorporation of [^{14}C]-choline into cellular LPC on the order of 200% and 250%, respectively, compared to control.

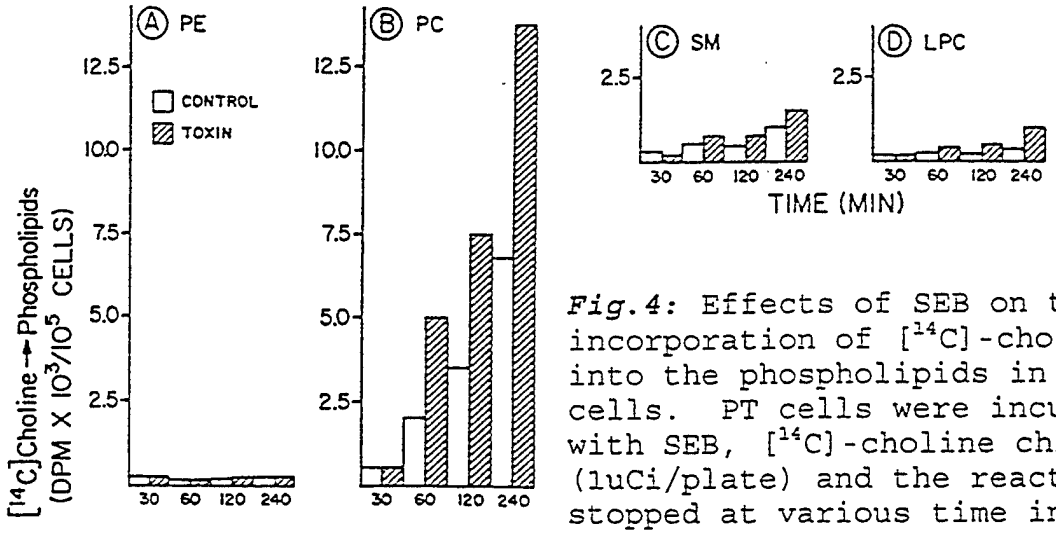

Fig.4: Effects of SEB on the incorporation of [^{14}C]-choline into the phospholipids in PT cells. PT cells were incubated with SEB, [^{14}C]-choline chloride (1uCi/plate) and the reaction stopped at various time intervals. The cellular lipids were extracted with hexane: isopropanol (3:2 v/v) and dried under N_2; the incorporation of [^{14}C]-choline into various phospholipids were measured as described in Materials and Methods. Open bars, control; hatched bars, cells treated with SEB. The results expressed are means of three experiments, each conducted in triplicate.

Effects of SEB on the cellular levels of phospholipids- SEB produced a time-dependent increase in the cellular levels of phosphatidlycholine. Whereas, the cellular levels of PC were similar to control after 30 min of incubation with SEB, the levels of PC increased 200-270% at 60 to 240 min after

incubation with SEB. The levels of SM increased about 150%, compared to control, after 60 min of incubation with SEB and remained unchanged for up to 240 min. The cellular level of LPC in cells incubated with SEB remained similar to control for up to 2 hr of incubation. A modest 126% increase in LPC was noted when cells were incubated for 4 hr with SEB (data not shown).

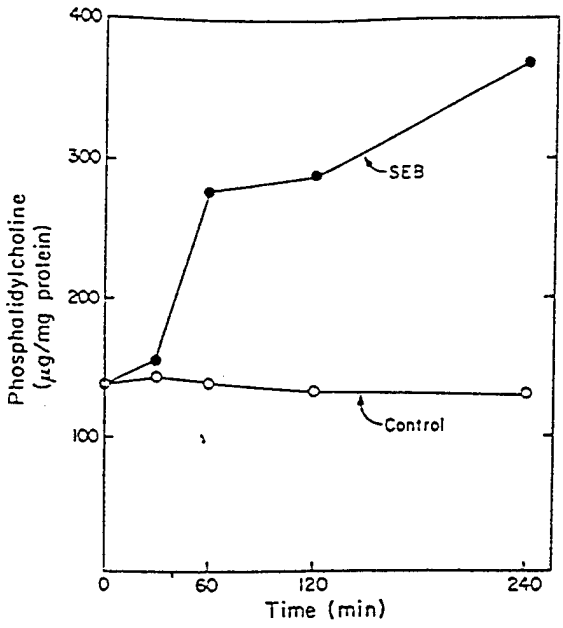

Fig.5: Effects of SEB on the level of phosphocholine in PT cells. PT cells were incubated with SEB and the reaction was stopped at various time intervals. The cellular lipids were extracted with hexane: isopropanol (3:2 v/v), dried under N_2, and measured as described in Materials and Methods. Three such experiments were pursued. Data (average value of triplicate analysis) from the third experiment are presented. The variation in the values were on the order of 5-10%.

Effects of SEB on CTP: phosphocholine cytidyltransferase activity- SEB produced a time-dependent increase in cytidyltransferase activity (Table 2). Although, CT activity increased only moderately after incubation of cells with SEB for 30 min, activity increased in the order of 2-fold, 4.5-fold and 4-fold after incubation with SEB for 60 min, 120 min and 240 min, respectively.

Table 2: Effects of staphylococcal enterotoxin-B on CTP:phosphocholine cytidyltransferase activity in cultured human kidney proximal tubular cells.

Incubation Time (min)	Cytidyltransferase Activity ($x10^3$ dpm/ mg protein/ 30 min)	
	Control	SEB
0	3.0	3.1
30	3.2	3.8
60	3.5	6.9
120	3.6	14.5
240	3.6	11.9

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